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(54) Vaccines for Combatting Septicemic Bacteria

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(57) Abstract

The bacteria expressing iron-regulated outer membrane proteins of which certain are siderophore or transferrin receptors can be used in a vaccinal preparation. Said bacteria are obtained from a culture in a medium in which the iron content is reduced to a level for obtaining an increased expression of said proteins, and in particular of receptors, sufficient to induce, when said bacteria are used in a vaccine, the formation of antibodies preventing the specific recognition of siderophores by their receptors.

VACCINE AGAINST SEPTICEMIC BACTERIA, SEPTICEMIC BACTERIA ANTIGEN PREPARATIONS, NEW BACTERIA AND VECTORS FOR THE PREPARATION OF THESE ANTIGENS OR VACCINES

5   This invention relates to bacteria belonging to genera including pathogenic bacteria and expressing large amounts of external membrane antigenic proteins regulated by iron.

          It also relates to processes for the production of these bacteria.

10   It furthermore relates to vaccines against septicemic bacteria containing as an active principle bacteria which express in large amounts external membrane antigenic proteins regulated by iron, or fragments of these bacteria or

15   antigens expressed by these bacteria.

          It furthermore relates to other bacterial, viral or other vectors allowing the expression of these antigenic proteins, as well as to vaccines containing these proteins as their active principle.

20   It furthermore relates to recombinant live vaccines, notably bacterial or viral vaccines

expressing these antigenic proteins in the vaccinated
organism.

It is known that apart from some lactobacilli
iron is a necessary nutriment for all living
forms including bacteria. These need iron to be
able to multiply in a host cell. The capacity
of the bacterium to multiply in vivo is an essential
factor of its virulence.

Although iron is present in large amounts
in a human body, the bacterium only has   a
very small amount of free iron at its disposal in
order to multiply.

Indeed by far the greater part of an
animal host iron   is intracellular (in the form
of ferritin , haemosiderin or heme) and therefore
its access is difficult. The small amount of iron
which is present in the body fluids only exists
in the form of extremely stable complexes, which
are principally made up of two iron chelating glycopro-
teins : transferrin in the plasma and lactoferrin
in the secretions. The existence of these glyco-
proteins strongly, but in a reversible manner,
linking the iron, is necessary in order to allow
its use by the cells, preventing its precipitation
in the form of ferric hydroxide.

The plasma contains iron complexes
in the form of haptoglobin-heme, ceruloplasmine,
ferritin , lactoferrin and transferrin.

The major part of the iron is transported
by transferrin s.   Three major classes of transferrins
may be recognized : seric transferrin , lactoferrin
and ovotransferrin .

The transferrin captures about 95% of the
iron in the plasma and its saturation rate
is only about 35% in the healthy individual.

Lactoferrin has a very small iron
saturation rate and keeps its chelating properties
in a wide pH range ; its presence in all secretions
of the organism, that is to say at the level of

5   potential microbial invasion sites, imposes a
wider restriction for iron in these places than
elsewhere in the organism.

The complexation of iron to glycoproteins
results in that only a very small concentration

10 of free ferric iron (10^{-19}M) remains, this being
quite insufficient to allow a normal growth of
bacteria.

In order to acquire the iron they need
to multiply in the host, bacteria have a number

15 of means available.

It seems that some microorganisms may
obtain their iron by a mechanism implying direct
interaction between the bacterial cell surface
and the protein linking the iron in the host.

20 However, this direct acquisition mode only affects
a very limited number of species. Most bacteria,
pathogenous or not, react to the lack of availability
of the iron in a host/in some aerobic environments,
by producing iron chelating compounds called siderophores.

25 Siderophores are made up by molecules
having a small molecular weight forming specific
complexes with a very high affinity for the ferric
ion. Their biosynthesis is regulated by the iron
and their function is to supply the bacterial

30 cell with iron.

These siderophores possess an extremely
high affinity for the ferric ion (their association
constant is about 10^{30}M^{-1}) which allows them to

displace the iron associated with the host protein or to solubilize the ferric iron which is precipitated in the form of hydroxide.

Most of previously identified siderophores belong to two chemical classes : phenolates-catecholates (deriving from 2,3-dihydroxybenzoic acid) and hydroxamates (deriving from hydroxamic acid).

The better known among siderophores belonging to the class of phenolates is the entero-bactin which is excreted by the bacteria belonging to the genera _Escherichia_, _Klebsiella_, _Salmonella_ and _Shigella_. This enterobactin is made up of a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine and is the chemical compound having the highest known affinity with the ferric ion ($Ka = 10^{52} M^{-1}$).

Several enteric species synthetise another hydroxamate siderophore, aerobactin. This siderophore is particularly synthetised by septicemic or invasive _Escherichia coli_ strains having a type Col V plasmid, or by _Salmonella typhymurium_ and _Shigella_.

This biosynthesis of siderophores by bacteria is associated with the production of proteins at the outer membrane, some of these proteins behaving as receptors for siderophores, as well as mechanisms allowing iron transportation and release inside the bacterium.
The common characteristic of these proteins which are formed in the outer membrane, and are often called "IROMP" meaning Iron Regulated Outer Membrane Protein, is a size between 70 kDa and 90 kDa, and their synthesis as well _in vitro_

in a restricted iron environment as _in vivo_ during
infection.

The outer membrane proteins, or siderophore
receptors, are therefore the ·cond element of
5   systems characterized as having a high affinity
for the bacterial intake of iron (the first
element being made up by siderophor es).

Apart from these high affinity systems,
many bacteria possess low affinity transportation
10   systems which allow them to use ferric hydroxide
in polymerized forms.

The absorption mechanisms for iron
have been particularly studied with _Escherichia_
coli which is the best genetically known micro-
15   organism.

The high affinity iron transportation
endogenous system in _E. coli_ uses the siderophore,
enterobactin. Enterobactin is synthetised and
excreted in the medium when _E. coli_ is placed
20   in a restricted iron environment. The ferric entero-
bactin complexes are then taken up by the outer
membrane (81 kDa Fep A protein) and transported
to the cytoplasm. When internalized the iron is
freed by ferric enterobactin hydrolysis, then
25   reduced to ferrous iron.

E. coli's enterobactin system comprises
at least thirteen genes. Seven genes (ent) are
involved in the biosynthesis of the siderophore,
and five genes (fep) code for transportation
30   proteins.

Apart from the enterobactin system,
E. coli septicemic strains excrete and carry an

hydroxamate siderophore, aerobactin.

It has been discovered in 1979 by P.H. WILLIAMS (37) that some Col V type plasmids carried genes for the aerobactin siderophore and its receptor located in the outer membrane and called Iut A protein (74 kDa protein).

Although the aerobactin has an association constant with the ferric ion which is lower than that of enterobactin, it however has structural properties increasing its capacity to take up the iron linked to the transferrin or to the lactoferrin.

After this was demonstrated in 1979 by P.H. WILLIAMS, many studies have shown that the aerobactin's iron transportation system played a major part in the virulence of pathogenic strains of E. coli and many other bacteria (GRIFFITH and al. (13)).

The presence of the aerobactin siderophore strongly favours the virulence of pathogenic strains.

Although aerobactin is less powerful than enterobactin as a chelating agent, it is active in much more varied environment conditions (enterobactin is very sensitive to oxidation and pH variations). Aerobactin therefore confers a higher degree of adaptation to the bacterium.

Besides, aerobactin is a better bacterial growth stimulator, and it seems that it is much more quickly excreted than enterobactin , probably because of a preferential genetic induction when E. coli is grown in the presence of a chelating agent.

With the aerobactin operon, the bacterium
acquires an extremely efficient iron transportation
system with a minimum number of additional genes,
or only four genes for the synthesis of aerobactin
which is a small simple siderophore, and one gene
which codes for the outer membrane receptor. Indeed
the other genes necessary for the transportation
of hydroxamates are inherently present in all
Escherichia coli.

The expression of all genes coding
for membrane proteins, siderophore receptors and
corresponding siderophores is regulated by a single
protein, Fur, which acts as a repressor when the
iron is available in sufficient amounts. The central
regulation is superimposed on an individual modulation
which regulates the expression of each system
according to the state of the environment.

Some authors have grown bacteria,
so as to increase the expression of IROMPs in
environment with an iron deficiency with the help
of chemical chelators, such as α, α' dipyridyl (A.
BINDEREIF et al) The cloacin receptor of Col V-
bearing Escherichia coli is part of the Fe 3+
aerobactin system, J. Bacteriol., 1982, 150, 1472-
475 ; C. MAROLDA et al. : Flanking and internal
regions of chromosomal genes mediating aerobactin
iron uptake system in enteroinvasive Escherichia
coli and Shigella flexneri, J. General Microbiology,
1987, 133, 2269-2278 ; A. BINDEREIF et al. :
Cloning of the aerobactin-mediated iron assimilation
system of plasmid col V, J. Bacteriol., 1983,
153, 1111-1113 ; De LORENZO et al. : Aerobactin
biosynthesis and transport genes of plasmid col V -
K 30 in Escherichia coli K 12, J. Bacteriol. 1986,

165, 570-578 ; P. WARNER et al. : col V - plasmid-specified aerobactin synthesis by invasive strains of _Escherichia coli_, Infection and Immunity, 1981, 33, 540-545).E. F. GRIFFITHS et al. have shown in   : Synthesis of aerobactin and a 76000 Daltons iron-regulated outer membrane protein by _Escherichia coli_ K-12 - _Shigella_ _flexneri_ hybrids and by enteroinvasive strains of _Escherichia coli_, Infection and Immunity, 1985, 49, 67-71, that enteroinvasive strains of _E. coli_ produce aerobactin and a 76 K outer membrane protein when grown in a reduced iron environment in the presence of ovotransferrin.

The recently acquired knowledge on the iron absorption systems of bacteria has allowed one to explore new ways of fighting pathogenic bacteria.

It has been suggested to synthetize siderophore analogs which are toxic for the bacterium and may deceive the iron transportation systems in order to penetrate into the bacterial cell. But these synthetic chelators have a lower affinity for the iron (III) than natural siderophores, and they are unable to displace iron in transferrins.

ROGERS has suggested to form complexes between aerobactin and trivalent metal ions in order to use them as antimetabolites towards the enterobactin -Fe^{3+} natural complex. Only complexes formed with scandium (Sc^{3+}) and indium (In^{3+}) have some antibacterial activity (ROGERS et al. (26) : ROGERS (27)).

It has also been suggested to adsorb phenolate type siderophores, which are aromatic

molecules, on some seric proteins, which then
play the part of carrier molecules, this allowing
the induction of specific antibodies against
the siderophore.

5 Thus, BYERS (5) has described a vaccine
against/the phenolate siderophore which is produced
by Aeromonas hydrophila (a bacterium which is responsible
for human and fish septicemia), which has been
assayed with fish. The siderophore is covalently
10 coupled with human or bovine albumine. Fish which
are immunized with these preparations generate
antibodies reacting against the siderophore. It
is, however not specified if the antibodies which
are formed are able to neutralize the siderophores.
15 One has also tried to prevent the bacteria
from taking up siderophores with antibodies that
would be specifically directed against the siderophore
receptors.

BOLLIN et al. (4) report the results
20 of a study indicating some passive immunization
with antibodies against the outer membrane proteins
regulated by iron, thus protecting turkeys from
an Escherichia coli septicemia.

However, all efforts towards the elaboration
25 of an efficient vaccine have, until now, failed
because of the difficulties in having a bacterium
express membrane proteins which are regulated
by iron at a sufficient level in the bacterial
culture.

30 This invention allows one to overcome
this difficulty by suggesting the use in a vaccine
as an active principle, of bacteria expressing
in large amounts membrane proteins which are

regulated by iron and more particularly, siderophore receptors at a sufficient level to induce the development of antibodies which prevent the specific recognition of siderophores by their receptors.

An aim of the invention is to supply bacteria expressing outer membrane proteins regulated by iron (IROMPs), which may be used as protective antigens.

Another aim of the invention is to suggest the synthesis of outer membrane proteins regulated by iron of septicemic bacteria by genetic recombination.

Another aim of the invention is to supply large amounts of IROMPs, notably Iut A and Fep A proteins, siderophore receptors, aerobactin and enterobactin in _Escherichia coli_ and other families, through synthesis of these proteins by a genetic recombination.

Another aim of the invention is to supply vaccines containing as an active principle, bacteria or fragments of these bacteria having in their outer membrane large amounts of IROMPs and notably Iut A and Fep A proteins, as obtained by genetic recombination or by other processes.

Another aim of the invention is to supply vaccines containing as an active principle IROMPs, for example, Iut A and/or Fep A proteins or antigenic preparations incorporating these proteins.

This invention therefore uses bacteria expressing outer membrane proteins regulated by iron and some of which are siderophore receptors, which may be used in a vaccine preparation.

Bacteria according to the invention
are charact... ...zed in that they express larger
amounts of t... ...se outer membrane proteins, and
more particu... ...rly, transferrin receptors, notably
siderophore ...ceptors, to induce, when these proteins
are used in ...vaccine, the generation of antibodies
preventing t... specific recognition function by
the receptor... and thus putting an end to the
iron supply ...f the pathogenic bacterium.
...teria which are used are preferably
enterobacter ...and they preferably excrete enterobactin
and/or aero... ...tin siderophores.
...teria are preferably chosen among
the group m... ...up by _Escherichia_ _coli_, _Klebsiella_,
Salmonella ...himurium, _Shigella_.
...eria preferably excrete together
the aeroba... ...and enterobactin siderophores.
...ording to the invention and according
to a first ...odiment thereof, bacteria are obtained
by growing ...turally existing strains or strains
that may b... ...und in laboratories or collections
in a minim... ...edium wherein the availability of
iron is rec... ...d to a level allowing a satisfying
higher expr... ...ion of/the membrane proteins. The culture
is prefera... ...grown in presence of a strong iron
(III)chelat... ...g protein such as lactoferrins, these
being chela... ...s which advantageously establish
an iron sho... ...ze with the same characteristics
as those th... ...are to be found _in vivo_.
...ct of the invention is also a process
for produc... ...uch bacteria to be used for the
preparation... ...vaccines, characterized in that

said bacteria are grown in a culture medium containing an iron (III) chelating protein such as transferrins, and notably lactoferrins.

As a minimum medium, one can use that which is described for instance by SIMON and TESSMAN (30).

However, this embodiment remains difficult to apply, because with the iron chelators which are generally used one must sufficiently reduce the iron content in the culture medium so as to obtain a sufficient expression of the outer membrane proteins. Often, small amounts of iron remain, preventing the expression of membrane proteins (iron from the fermentor, for instance or from pipes which are, generally, made of stainless steel). One must then resort to comparatively complex processes in order to lower the iron content to a level allowing the expression of the external membrane proteins of bacteria, and their application is costly.

According to a second embodiment of the invention, which is also the preferred embodiment, bacteria expressing in large amounts the outer membrane proteins, siderophore receptors, are transformed by recombinant plasmids.

Indeed, he advantages of synthetizing the outer membrane proteins, siderophore/or transferrin, receptors through genetic recombination, are many :

- it allows one to generate an important expression of these proteins, whatever the iron concentration in the culture medium,

- it allows one to study immune reactions directly aiming at these proteins, while excluding

any other constituent of the original strain,

 - it represents the cheaper solution
for the expression of membrane proteins in an
environment wherein iron is always present (fermentors,
pipes and sundry stainless steel equipment).

 If the applicant more particularly
aims at the synthesis of Iut A and Fep A proteins,
aerobactin and enterobactin siderophore receptors
of E. Coli, it can be understood that the below
described genetic recombination methods will
apply by analogy with the synthesis of membrane
proteins (IROMPs), siderophore receptors, aerobactin
and enterobactin or tranferrins from pathogenic
bacteria other than E. coli.

 The invention therefore also relates
to the preparation of E. coli Iut A and/or Fep A
proteins by genetic recombination.

 The Iut A protein may be synthetized
by a process according to which, in particular :

 - one isolates the plasmid or chromosome from
    Salmonella. Shigella or Klebsiella pathogenic
E. coli strains, bearing the aerobactin operon,

 - one separates from the plasmid or
chromosome a fragment containing the iut A gene,

 - one links said fragments with a
cloning vector,

 - one inserts the clones having inte grated
the iut A gene in an expression vector (for example
GTI 001 plasmid),

 - one then expresses the Iut A protein
by growing the clones.

The Fep A protein is obtained :

- by isolating from a plasmid (for example pMS 101 plasmid built by LAIRD and YOUNG (19)) or from a bacterial chromosome, E. coli, Salmonellae or Klebsiellae, a fragment bearing the fep A gene,

- by cloning said fragment in a cloning vector,

- by inserting the fep A gene in an expression vector, preferably in a vector which is used for the expression of Iut A protein (GTI 001 plasmid),

- by the expression of the Fep A protein by growing the clones.

The expression vectors for Iut A and/or Fep A proteins may be bacteria and one prefers to use E. coli whose expression systems are best known. One may however also use other vectors, notably viral vectors or vectors made up of yeast, and which can be built by specialists.

Bacterial clones expressing Iut A and/ or Fep A proteins may be multiplied in an appropriate medium at a sufficiently low temperature so as to prevent or limit the expression, generally below 32°C. The expression is then induced by rising the temperature, for example to 42°C during about 4 hours, so as to induce the expression of iut A and fep A genes.

One therefore obtains bacteria integrating Iut A and Fep A proteins as well as their proIut A and proFep A precursors, these having the shape of large size cytoplasmic inclusions.

These bacteria as used in a vaccine,
as an active principle, induce the generation
of antibodies directed against Iut A and Fep
A proteins, preventing recognition by these proteins
5   of their respective siderophores aerobactin and
enterobactin, this therefore strongly reducing
the iron supply to the bacterium and blocking
its multiplication.

The invention therefore also relates
10   to vaccines containing as an active principle
recombinant bacteria expressing external membrane
proteins regulated by iron.

The invention more particularly relates
to vaccines containing as an active principle :
15   recombinant bacteria or fragments of these bacteria,
notably fragments of membranes integrating Iut
A and/or Fep A proteins or their precursors, proIut
A and/or proFep A ; or again Iut A and/or Fep
A proteins and/or their precursors, for example,
20   extracted from cytoplasm or extracted from the outer
membrane of recombinant bacteria.

In another embodiment, the invention
relates to vaccines containing as an active principle :
bacteria which are homologous to septicemic bacteria,
25   or fragments of these bacteria, grown in a medium
with restricted iron supply, and which integrate
in larger amounts Iut A and/or Fep A proteins
                                    or
and/or their precursors; /Iut A and/or Fep A proteins
(and/or their precursors) being suitably extracted.

30   The latter vaccines are preferably
prepared from bacteria which are grown in a medium
containing a strong protein type iron (III) chelator,

notably transferrin, lactoferrin or ovotransferrin.

The invention would be better understood on reading the following specification , referring to the appended drawings, wherein :

Figure 1 is the protein profile as obtained by electrophoresis of a clone expressing Iut A protein,

Figure 2 is a protein profile as obtained by electrophoresis of a clone expressing Pep A protein.

The abbreviations used in the following specification have the following meanings :

Amp^r ampicillin   resistant

Clo^s cloacin   sensitive

dATP   deoxy-adenosin-triphosphate

EDTA   ethylene-diamine-tetraacetic acid.

Ent   enterobactin

E.O.P.S.   without specific pathogenic organisms

IPTG   isopropyl-ß-thiogalactopyranosid

kpb   bases kilopair

LB   luria broth

OMP   Outer-membrane protein

PAGE   polyacrylamide   gel electrophoresis

pb   bases pair

PBS   phosphate buffered saline

SDS   Sodium dodecyl sulfate

ST   Simon and Tessman

TEMED   N,N,N',N'-tetramethylene diamine

Tris   tris-hydroxy-aminomethyl-methane

tet^r tetracyclin   resistant.

MATERIALS AND METHODS

I. MATERIALS

1. Strains

Table I recapitulates the various strains. The pathogenic strains used are septicemic strains from calves or chicks and are to be found in the RHONE-MERIEUX strain collection.

The host strains .. used for cloning, sequencing and expression are all derived from Escherichia coli K 12.

These strains may be easily replaced by other wild septicemic strains or laboratory strains;

2. Plasmids

Origin and characteristics of plasmids used for cloning and expression are presented in Table II.

3. Media

SIMON and TESSMAN minimum medium (30)

Contents :

NaCl	5.8	g
KCl	3.7	g
$CaCl_2$, $2H_2O$	0.15	g
$MgCl_2$, $6H_2O$	0.10	g
NH_4Cl	1.10	g
Na_2SO_4	0.142	g
KH_2PO_4	0.272	g
Tris	11.20	g
H_2O qsp	1000 ml	pH 7.4

The only carbon source is sodium succinate added to a final concentration of 10 g/l.

In order to establish a limitation to the iron in this medium, one adds ovotransferrin (SIGMA) at a final concentration of 250 ug/ml (One may have concentrations above 500 ug/ml).

5　　The iron-rich control medium is obtained by adding $FeCl_3$, 6 H_2O (MERCK) at a final concentration of 40 uM.

M9 MANIATIS minimum medium (29)

Na_2HPO_4	6.0	g
KH_2PO_4	3.0	g
NaCl	0.5	g
NH_4Cl	1.0	g
H_2O qsp	1000 ml	pH 7.4

to this basic medium are added :

Mg SO_4 1M	2 ml/1000 ml	
glucose 20 %	10 ml/1000 ml	
$CaCl_2$ 1M	0.1 ml/1000 ml	

- LB rich medium (MANIATIS and al. (23))

bactotryptone	10 g	
Yeast extract	5 g	
NaCl	5 g	
H_2O q.s.p.	1000 ml	pH 7.4

BTS rich medium (BIO MERIEUX)

Biotryptase	17	g
Biosoyase	3	g
NaCl	5	g
K_2HPO_4	2.5	g
glucose	2.5	g
H_2O qsp	1000 ml	pH 7.3

- BHI rich medium (heart-brain BIO MERIEUX)

Calf brain infusion	200 g	
ox heart infusion	250 g	

Bio-gelytone	10	g
NaCl	5	g
Na$_2$HPO$_4$	2.5	g
glucose	2.0	g
H$_2$O qsp	1000 ml	pH 7,4

- "M9 SP" rich medium for the expression medium :

SP (tryptone 3.2% : yeast 2%)	100 ml
M9 (6.6 x concentrate filtered on 0.22 um filter (Millipore)	15 ml
MgSO$_4$ 100 mM	1.5 ml
FeCl$_3$ 0.1 mM	1.5 ml
vitamin B1 (5% solution)	1.5 ml

The solid media have the same composition as that of the corresponding liquid media and contain 12 g agar per liter of the medium.

Antibiotics are used in solid and liquid media at the following final concentrations :

| Ampicillin | 25 ug/ml |
| tetracyclin | 12.5 µg/ml |

isopropyl-1-ɔ-D-thiogalactopyranoside (IPTG) is optionally added at a final concentration from 0.05 mM to 0,4 mM.

Sterilization of liquid and solid media is made by autoclave at 120°C during 20 minutes.

Antibiotics, vitamin B1, sodium succinate M9 6.6X, MgSO$_4$, FeCl$_3$, IPTG and ovotransferrin solutions are made in the form of concentrated stock solutions, and sterilized by filtration on a filter having 0.22 um porosity (Millipore).

After sterilization, the growth media are kept at room temperature.

Antibiotics, IPTG and ovotransferrin

solutions are kept at -20°C.

Other solutions are kept at +4°C

II. METHODS

1. Bacterial cultures

Apart from clones with structures built
in the pGTI 001 expression vector, which are grown
at +30°C, all cultures are made at ÷37°C, while
stirring, during 18 hours.

Whenever necessary, bacterial growth
is estimated by measuring the suspension turbidity
at 600 nm, with the help of a BECKMAN DU 40 spectro-
photometer.

Cultures are usually made in a volume
of 2 ml after seeding with a colony. Cultures
in a more important volume (20 ml to 1000 ml)
are made by seeding to the 1/100th with a preculture
in stationary phase.

2. Sensitivity toward bacterocins

Productions of cloacin DF 13 and colicin
B are made with Enterobacter cloacae DF 13 and
Escherichia coli 1300 strains, respectively, according
to the process described by DE GRAAF (DE GRAAF
et al. (8) and (9)).

These strains are grown at +37°C, in
a BHI medium, until the optical density reaches
0.5 (1 cm, 600 nm).Mitomycin C.is then added to
the growth medium, so as to reach a final concentration
of 1 ug/ml, which allows one to induce the synthesis
of bacteriocins. This culture is prolonged during
6 hours, at +37°C, until lysis phase. Bacterial
bodies are centrifugated (8000 g, 30 nm, +4°C)
and the supernatant is harvested. Ammonium sulfate
is then slowly added at +4°C, until the concentration

reaches 365 g/litre.

The supernatant is taken up in 0.05 M
phosphate buffered   pH 7.0 and dialysed against
several succeeding baths of this buffer. The dialysate
is filtrated over 0.22 μm (Millipore) and kept
at -20°C.

About 10^9 bacteria of the clone under
study are spread on LB agarose containing the
appropriate selection antibiotic. When the deposition
liquid is completely absorbed, one places at the
center of the Petri dish 75 μl of the bacteriocin
solution. When this drop has itself dried, the
Petri dish is placed in an incubator (+30°C or
+37°C, as the case may be) during 18 heures. Clones
which present a growth inhibition around this
deposition have became bacteriocin-sensitive.
Clones which resist the toxic effect of bacteriocin
on the contrary exhibit a uniform bacterial mat.

3. Preparation of antisera directed against
the outer membrane proteins regulated by iron

The protocol which is used is a repetition
of that which is described by BOLIN and JENSEN
(4).

The outer membrane proteins regulated
by iron are separated by polyacrylamide gel
preparative electrophoresis with sodium dodecyl
sulfate added.

When   the gels are coloured, the strip
containing the IROMP to be used for the immunization
is cut off, then comminuted with distilled water,
by passing through several needles having smaller
and smaller diameters.

This ground product is injected to E.O.P.S.

(29) : Triton X-100 2% ; $MgCl_2$ 10 mM ; Tris/HCl
50 mM pH 8.0.

Incubation is carried out during 30
minutes at room temperature, while shaking every
five minutes. During incubation the cytoplasmic
membrane proteins are preferentially solubilized
by Triton X-100. Outer membranes are collected
through a new ultracentrifugation (111,000 g,
60 minutes, +4°C). The obtained pellet is thrice
washed in distilled water, finally resuspended
in 1 ml distilled water and frozen at -20°C for
storage.

6. Proteins dosage

The membrane extract protein concentration
is measured by a colorimetric method derived from
that published by LOWRY et al (20).

. To 0.5 ml protein solution to be dosed
are added 2.5 ml of the following solution :

1% $CuSO_4$ solution   1 ml

2% sodium tartrate
solution   1 ml

2% sodium carbonate
solution in 0.1N NaOH   q.s.p.

After incubating 10 minutes at room
temperature, 0.25 ml 50% Folin reactant (Merck) is
added.

Incubation is carried out for 30 minutes
at room temperature, and the optical density of
the blue color   which has   evolved is measured
at 779 nm.

Protein concentration of samples   is
determined with a standard interval prepared with
bovine serum albumin .

The optical density is proportional
to the protein concentration in an interval
of 5 to 200 μm/ml.

7. <u>Techniques for the analysis of the</u>
<u>outer membrane protein composition : polyacrylamide</u>
<u>gel electrophoresis under denaturating conditions</u>

Polyacrylamide gels are prepared according
to the characteristics described by LUGTENBERG
et al. (21).

The alignment gel has the following
composition :

acrylamide-bisacrylamide (30/0,8 p/p)
5% ; Tris/HCl 130 nm pH 6,8 ; SDS 3,5 mM ; ammonium
persulfate 44 mM TEMED 8 mM.

The séparation gel has the same composition
as the alignment gel, except for the acrylamide/bis-
acrylamide concentration (8 or 10%) and the Tris/HCl
buffer 380 mM pH 8.8 concentration.

The migration buffer used has the following
composition :

glycine   14.4 g

Tris   3.0 g

SDS   1.0 g

H_2O q.s.p. 1000 ml   pH 8.3

Extracts to be analyzed or purified
by electrophoresis are diluted in at least an
equal volume of the following dissociation buffer :
Tris/HCl 100 mM pH 6.8 ; glycerol 20% ; SDS 70 mM,
3-mercapto-ethanol 100 mM ; bromophenol blue 75 μm.

The thus diluted extracts are heated
to 100°C during 5 minutes.

In order to analyze the outer membrane
protein composition 30 to 50 ug proteins are deposited
in each well.

For prep. .tive electrophoreses,   as
much as 2 mg protei.   are deposited in the sole
preparative wel
                Migratic   s carried out at +14°C during
5   5 hours at 160 V or   hours at 60 V (Vertical
gel LKB apparatus).   order to increase the resolution
of the outer membra.   proteins regulated by iron,
some electrophores·   have been under a voltage
of 100 V during 16 . :rs.   At the end of the electrophoreses,
10   proteins are fixate . .nd coloured during 30 minutes
at room temperatur.   :h 1.2 mM Coomassie blue
in a (50:10:50 v/v   methanol/acetic acid/water
mixture. The unfix.   . colour is eliminated with
several succeeding   :15:145 v/v/v) methanol/acetic
15   acid/water baths at . c.   once decolorated, the
gel is photographeu.   ·d dried.
                The out: rane protein profiles
for each strain ma   . be analyzed by densitometry
(LKB ULTROSCAN las   ·sitometer).
20   8. Dete   and analysis of anti-
IROMPs antibodies

                The pr   · of specific anti-IROMPs
antibodies is inve.   ·ed with the microplate
ELISA technique (E.   .. and PERLMANN (10) ; COULTON
25   (7)). Antigens whi   · coupled to the solid
phase are fractio: ·h are very much enriched
in proFep A prot   proIut A protein.
                The· rev   . of anti-IROMPs antibodies
is made with a ra.   :ti IgG conjugate (or chicken
30   anti IgG) coupled   roxidase (Nordic). The
substrate used is   .ophenylene-diamine. Readings
of optical densiti·   re made at 492 nm.

-"Western-blotting" technique

Proteins which are separated by polyacrylamide gel electrophoresiswith SDS are transferred on a polyvinylidene fluoride membrane (PVDF 0.45 um Millipore) according to the method described by TOWBIN et al. (34).

Transfer is made under 24 V during one hour with a BIOLYON apparatus using the following anodic and cathodic buffers :

anodic buffer	cathodic buffer
Tris 0.3 g	Tris 0.3 g
glycine 1.44 g	glycine 1.44 g
methanol 100 ml	SDS 0.1 g
H_2O qsp 500 ml	H_2O qsp 500 ml

After transfer, the PVDF membrane is saturated during one hour at +37°C in PBS buffer containing 1% skimmed milk.

The membrane is then cut into strips corresponding to electrophoresis tracks.

Sera to be studied are diluted into PBS buffer containing 1% skimmed milk, then contacted with membrane strips, 4 ml diluted serum per strip.

After incubation for one hour at +37°C, while gently stirring, three 20 minutes washings are made in PBS buffer containing 2% skimmed milk at room temperature.

An anti IgG conjugate coupled to peroxidase, diluted to the 1/1000 in PBS containing 1% skimmed milk is added at a rate of 3 ml per strip.

After incubating one hour at +37°C while gently stirring, 3 20 minutes washings are made at room temperature in PBS buffer.

The diaminobenzidine substrate, diluted
to 0.1% in a physiological water at pH 7.15 to
which 30 volumes 0.1% H_2O_2 have been added,
is then added to the whole. One then notices
(after 5 to 20 minutes) brown coloured strips
around proteins which are recognized by the antibodies
in the serum under study.

The membrane is then washed in distilled
water and dried up.

9. Plasmid DNA preparation method

Large plasmids contained in pathogenic
strains are extracted according to the method
published by KADO et al. (16).

Plasmids obtained during the various
stages of cloning, undercloning, and construction
in the expression vector are extracted according
to the method of BIRNBOIM (BIRNBOIM and DOLY)
(17). Plasmid DNA obtained after preparative extractions
made with one of these methods is purified on caesium
chloride gradient (MANIATIS et al. (23)).

Whence purified, the plasmids are taken
up in Tris 10 mM ; EDTA 1 mM pH 8.0 buffer so
as to reach a final concentration of 1 μg DNA/μl,
and frozen at -20°C for storage.

10. DNA analysis and modification methods

All methods used for cloning, DNA digesting,
restriction fragment analysis in agarose gel,
modification of the ends of restriction
fragments, hybridation with a radioactive probe
after transfer of DNA on nitrocellulose membrane,
are described by MANIATIS (MANIATIS et al. (23)).
DNA has been sequenced and oligonucleotides have
been synthetized according to special methods.

11. TN   quencing

Genes .   sequenced are subcloned
in M13 mp 18 and m   vectors (YANISCH-PERRON
et al. (38)) and   uenced according to the
chain end method o/   deoxynucleotides (SANGER
et al. (29)). Lab   ing of the various chains
is made with ^{25}S   r (AMERSHAM).

Sequen   g proper is made with reactants
and sequencing ki   enzymes Amersham and Sequenase
(USB). Electropho   ses in a polyacrylamide gel
with urea are mad   in a Sequi-Gen (BioRad) apparatus.

Sequen   ng data are processed with
Microgénie (Beck   software.

12.   onucleotides synthesis

The v   ous oligonucleotides which
are necessary t/   e constructions the expressing
vectors or to th   utageneses are synthetised
according to th.   yano-ethyl-phosphoramidites
method on Appli   Systems 381-A apparatus.

Thes   ligonucleotides are directly
used after depr   tion and precipitation with
ethanol.

13.   cted mutagenesis method

Muta   sis is made according to the
method describe   ECKSTEIN (TAYLOR et al. (33) :
NAKAMAYE and EC   IN (24)) with the directed
mutagenesis k.   old by the Amersham Company.

RES

1.   TION AND ANALYSIS OF THE A
                   S

The   ence of the aerobactin opero
has been sear   on plasmids born by 1534,
1537 and .

After purification on caesium chloride
gradient, these plasmids are digested by Bam HI,
Hind III, Pvu and Sal I restriction enzymes (Boehringer).
The various restriction fragments of each plasmid
are separated on 0.8% agarose gel and transferred
on a nitrocellulose membrane according to the
"Southern blot" (SOUTHERN (31)) method. These
fragments are then hybridated with two radioactive
probes (labelled with 32p by displacement of cut)
prepared from pABN1 plasmid restriction fragments
bearing the aerobactin operon of pCol V-K30,
(BINDEREIF and NEILANDS (2)). It has been found
that the gene coding for the IutA receptor exists
on all plasmids on a 6.6 kb Bam HI-Bam HI restriction
fragment. These results are a confirmation of
the authors' work of undercloning this gene from
an equivalent PCol V-K30 fragment (KRONE et
al. (17)).

- Cloning of plasmid DNA fragments
bearing the IutA gene

Plasmids of 15393, 15972 et 16003
strains are digested by Bam HI restriction enzyme.
After electrophoresis on 0.8% agarose gel, the
gel strip containing the 6.6 kb fragment of each
plasmid is cut and the DNA is electroeluted.

The three 6.6 kb Bam HI-Bam HI fragments
are ligated separately and the paT 153 vector digested
by Bam HI. The three ligation mixtures are used
to transform competent HB 101 bacteria (table
IV).

The DNA of ampicillin-resistant, tetra-
cycline sensitive clones is extracted by the
method of BIRNBOIM and DOLY and digested by Bam
HI in order to check the size of the insert.

Clones having integrated the 6.6 kb fragment are
selected on the basis of their sensitivity to
cloacine DF 13.

For each starting plasmid a cloacine
sensitive clone is kept for analyses and further
build ups. They are the following clones :

Strain 15393   clone HB 101 p 5 - 15
Strain 15972   clone HB 101 p P - 13
Strain 16003   clone HB 101 p 4 - 18

- Clones analysis

A study of the expression of iut A
receptor.

Having been able to select the desired
clones with a cloacine sensitivity test shows
that there is an expression of the receptor to
cloacine and aerobactin. Expression of the iut
A gene born by the 6.6 kb Bam HI-Bam HI fragment
probably depends from a weak promoter situated   on this
fragment (KRONE et al. (17)) and does not depend
on the iron concentration as does the main promoter
of the operon aerobactin. Indeed, the sensitivity
to cloacine is demonstrated with a culture on
iron-rich ampicillin-LB gelose.

In order to study the expression level
of the iut A receptor, the various clones are
grown in LB-ampicillin medium (for one night at
37°C) with or without ovotransferrin (500 µg/ml).

The outer membrane proteins of each
of the three clones are extracted and analyzed
on polyacrylamide gel with SDS.

Whatever the growth conditions, it
is possible to note a 76 kDa supernumerary protein

expression in cloacine sensitive clones.

- Restriction maps

Plasmid DNA of clones 4-18, 5-15 and
P-13 is extracted in large amounts and purified
on caesium chloride gradient.

Restriction maps during the three 6.6
kb Bam HI-Bam HI fragments which have been cloned are drawn
with Bgl II, Bst E II, Cla I, Eco RI, Kpn I,
Pst I, Pvu II and Sma I.

These three maps are totally identical
and correspond to the restriction map of the 6.6
kb Bam HI-Bam HI fragment of p ColV-K30 aerobactin
operon deduced from maps published by BINDEREIF
and NEILANDS (1,2) and KRONE et al. (17).

These four maps are represented in
table V.

- Sequencing of iut A genes

When comparing restriction maps for
the three 6.6 kb Bam HI-Bam HI fragments with
the restriction map as deduced from the sequence
of the iut A gene (KRONE et al. (18)), it appears
that the iut A gene is entirely to be found
on a 3.2 kb Bst E II-Bst E II restriction fragment
(table VI).

This fragment is isolated by electro-
elution, religated upon itself with T4 phage ligase
(Boehringer) and digested with several restriction
enzyme systems. The various fragments thus obtained
(size 150-600 pb) are isolated by Geneclean (Bio
101) and subcloned in the M13 mp 18 and mp 19
vectors previously digested with the appropriate
enzymes. The sequence of each subclone is then

determined according to SANGER's method with
the use of Amersham and Sequenase (USB, sequencing
kits.

Only the 3.2 kb Bst E II-Bst E II
fragment of clone P-13 has been entirely sequenced.
The two remaining iut A genes have been sequenced
between the Bgl II (1) site and the Eco RV (2396)
site.

The Bst E II-Bgl II region of clone
P-13 has been compared with the sequence of the
iuc D gene located just upstream of iut A
(HERRERO et al. (14)) and the Pvu II-Bst E II
region of this clone has been compared with the
sequence/IS1 element (OHTSUBO and OHTSUBO (25)).

These comparisons as well as the
comparisons of the three
iut A genes with the sequence of the iut A gene
of pColV-K30 are presented in table VII.

Analysis of these four sequences reveals
that the iut A gene was extremely well kept at
the molecular level. Apart from one or two bases,
the three iut A genes isolated from E. Coli strains
of animal origins are identical. Differences observed
with the sequence published by KRONE et al. (18)
are minimal.

The three iut A gene under study are
99.77% homologous to the iut A gene of pColV-K30.

Regions where differences have been
demonstrated are represented in table VIII. (Numbers
relate to the position of bases in sequences presented
in table VIII).

Two important regions are totally preserved :
the sequence coding for the signal peptide and
the consensus sequence (" Ton B box") typical
of the outer membrane protein receptors whose
5   function depends from Ton B.

The existence of four inserts in relation
to gene _iut A_ of ColV-K30 has been demonstrated
on each of the three sequenced genes. These inserts
trigger limited changes in the reading frame.
10 Thus, the primary structure of iut A proteins
coded by isolated plasmids of the strains under
study is a little larger (+ 8 amino acids) than
that of protein Iut A of p ColV-K30. The sequence
of isolated _iut A_ genes of the strains under study
15 codes for a 733 amino acid polypeptide comprising
a 25 amino acids signal peptide which is identical
to that of the Iut A polypeptide of strain ColV-
K30. The calculated mass of the mature protein
is 78097 daltons, which differs slightly from
20 the size observed on gel (76 kDa). However, the
changes in the primary structure are not sufficiently
important to alter the secondary structure and
the hydrophilicity profile.

2. EXPRESSION OF CLONED IUT A AND fep A
25   GENES

- Characteristics of the vector under
  study

The expression vector used is the
GTI 001 plasmid built up by the Mérieux Institute
30 genetics engineering laboratory.

The genes it bears and its restriction
map are presented in table IX.

This plasmid may be built up as follows :

Plasmid pBRTac, made up by pBR322 (Bolivar F. et al. Gene 2, 95-113 (1977)) propagating between HindIII and BamHI the promotor Tac (Ammann E. et al., Gene 25, 167, (1983)), has its XhoI site destroyed by the kleenow polymerase (also called "the kleenow") to give plasmid pBRTacX⁻. This plasmid is digested by NcoI, treated by the kleenow, then digested by AvaI, and its smaller fragment is ligated to the pMC9 fragment (Casadaban M.J. et al., Journal of Bacteriology, 143, 971-980 (1980)) digested by MstII, treated with the kleenow and then digested by AvaI bearing gene Lac i and the replication origin of pBR322.

The resulting plasmid (named pBRLaciX⁻) is digested by HindIII, treated with the kleenow and then digested by PstI, and the 2350 bases pair (or "pb") fragment is ligated to the 2300 pb fragment of pBRTac digested by EcoRI, and then treated with the kleenow, and digested by PstI, thus creating plasmid pBRTaci. The latter's 4406 pb fragment, obtained by digested with EcoRI and PstI is ligated with a 1688 pb fragment digested by EcoRI and PstI and derived from pBR322 sequences and bearing the pBR322's tetracycline resistance gene.

The obtained plasmid is called pBRTaciTet. The latest replication origin is separated by digestion with BamHI, and the remaining 2096 pb fragment is ligated to the 2033 pb fragment of pATI53 (Twigg A.J. & Sherratt, D. Nature, 283, 216-218 (1980)) digested by XhoI, thus creating plasmid pATTaciUri.

This plasmid is digested by EcoRI,
then treated with the kleenow, and digested by
AvaI and the 2704 pb fragment is ligated with a
fragment bearing promoter Pr and its thermosensitive
CI857 repressor of the 3076 pb Lambda bacterio-
phagederived from pCQV2 (Queen C. et al., J. Mol.
Appl. Genet. 2, 1-10 (1983)) by digestion by PstI,
treatment with the Mung Bean nuclease, and partial
digestion with AvaI. The resulting plasmid is
called pGTI001.

The replication origin of this plasmid
is under control of promoter tac, which allows
one to regulate the number of copies by growing
the bacteria in a medium containing various IPTG
(gene lac i inductor) concentration.

The gene to be expressed is placed
under control of phage CI 857 strong "Pr" promoter
(the phage's repressor being thermosensitive).
The ATG of the gene to be expressed is replaced
by the ATG of gene cro. This ATG is created by
partial Bam HI digestion of p GTI 001 followed
by digestion with Mung Bean nuclease so as to
obtain a blunt end.

The gene to be expressed is inserted
in phase (starting from codon number 2) between
the blunt ATG end and the XhoI site. An
ending signal for the transcription, placed just
downstream from the XhoI site, avoids the
production of too long messenger RNAs.

Other plasmids of this kind able to
express gene iut A (or fep A), are easy to obtain
or to build and one knows such plasmids wherein the gene
to be expressed is controlled by a promoter whose
repressor is thermosensitive.

- Construction of the expression vector of gene iut A

iut A genes isolated from strains 15972
and 16003 are cloned with their signal sequence at the level
of the expression site Bam HI (899) of p GTI 901.

To obtain a blunt 5' end, starting
with the second amino acid of the signal sequence
(this time methionine), a two strands synthetic
oligonucleotide is used to replace the region
comprised between the initiation ATG and the only
Acc.I site situated in 5' of the coding sequence.
The sequence of this oligonucleotide is presented
in table X.

The two complementary strands of this
oligonucleotide are synthetised and the double
strands form is obtained by heating to 90°C
in a NaCl 50 mM, Tris 10 mM, MgCl 10 mM, pH 7.5
buffer, of an equimolar mixture of the two simple
strands, followed by a slow cooling down to room
temperature.

The strategy which is followed in order
to insert gene iut A of strain 15972 (clone p
P-13) is presented in table XI.

With a not very satisfying yield. a modified strategy
is adopted to put in phase gene iut A of strain
16003. This new strategy uses a sub-cloning
of the Eco RI-Eco RI region of the intermediary
construct (step 4, table XII) in vector pSB 118.
This vector is a pUC 18 derivative. It has a
"polylinker" between two Eco RI sites. Sub-cloning of
the Eco RI-Eco RI fragment in this vector has thus
allowed one to put in phase gene iut A while avoiding
partial digestions. (Table XII).

Restriction map of the

GTI P-2 (gene iut A of strain 16003) and GTI
P-5 (gene iut A of strain 16003) expression
plasmids thus obtained are presented in table
XIII.

Clones obtained after renewed ligation
with the double strand "iut" oligonucleotide
are selected on the basis of the preservation
of site Acc I in gene iut A and the disappearance
of site Bam HI in p GTI 001. All clones presenting
this restriction profile have been controlled
at the expression of protein Iut A.

- Control of the expression of Iut A

Qualitative control

The selected clones are grown in M9
SP tetracycline medium with 0.4 mM IPTG at 32°C
(start of induction). The sensitivity of these
clones towards cloacine is examined by the above
described method.

Two clones out of 25 are positive for
the constructions GTI-iut 15972.

Three clones out of 6 are positive
for the constructions GTI-iut 16003.

The cloacine sensitive clones are
grown in 50 ml tetracyclin M9 SP medium containing
0.4 mM IPTG.

The culture is made at a temperature
of 30°C till the optical density reaches 1. Induction
of gene iutA expression is then made by continuing
the culture for four hours at ~42°C. The bacteria
are centrifugated and their total proteins are
analyzed by polyacrylamide gel-SDS electrophoresis.
This allows one to directly appreciate the importance

of Iut A protein production (figure 1). The analysis
of clone CMK 603 GTI P-2 reveals that protein
Iut A and its precursor represent, after induction,
25% of the bacterium's total proteins. The sole
Iut A protein represents 30% of the outer membrane
proteins.

- Construction of the expression vector
for gene fep A.

Characteristics of the initial clone
p MS 101

Following the results of CODERE and
EARHART (6) indicating that gene fep a is located
on a 6.3 kb Bam HI-Bam-HI fragment of plasmid
pMS 101 constructed by LAIRD and YOUNG (19), this
fragment is sub-cloned and vector pBR 322 digested
by Bam HI. The restriction map of the obtained
plasmid (P-1) appears similar to that of plasmid
pITS 1 (FLEMING et al. (11) -table XIV).

The publication of the sequence of
fep A (LUNDRIGAN and KADNER) (22) has enabled
one to precisely locate this gene on the restriction
fragment Ssp I-Stu I 2530 pb of plasmid P-1.

The following strategy is used to insert
gene fep A into p GTI 001.

An oriented mutagenesis is made in
terminal 5' region of the coding region in order
to transform sequence :

5'ATGAACAAG 3'

into a HpaI restriction site

MET ASN LYS

‒‒

G T T A A C A A G

This site is cut into blunt ended ends in the following manner GTT AA C. This allows direct ligation of gene fep A with the ATG end created in p GTI 001.

The mutagenesis is made according to the ECKSTEIN method from an oligonucleotide (table XV), after sub-cloning of 800 pb Ssp I-Eco RI fragment in the replicative form of phage M13 mp 19.

The mutated fragment is entirely sequenced in order to check that the sequence had not been modified elsewhere than at the desired site.

Details of integration of gene fep A are summarized in tables XVI and XVII.

The various clones obtained after ligating fragment HpaI-XhoI 2350 pb in plasmid GTI 001 are selected by the presence of a 1700 pb Eco RI-Eco RI fragment.

- Control of the expression of Fep A

Qualitative control.

The ligation mixture between the 2350 pb Hpa I- Xho I fragment and plasmid GTI 001 is used to transform competent bacteria RWB 18. This strain being fep A, it is colicine B resistant.

Clones having the desired restriction map (table XVII) are tested upon their sensitivity to colicine B.

One clone (RWB 18 GTI F-12) appears sensitive to the action of colicine B.

Quantitative control.

The expression of protein Fep A and its precursor is analyzed on polyacrylamide-SDS gel (figure 2). Clone CMK 603 GTI F-12 expresses

Pep A and its precursor in a very large amount
(20% of total proteins).

Protein Pep A represents 32% of the
outer membrane proteins.

- Physiological and morphological study
of clones expressing Iut A and Pep A

Growth potential

The growth potential of obtained clones
is tested at various growing temperatures in LB
tetracycline IPTG medium.

When seeded as a layer on LB tetracyclin
IPTG agarose 0.1 mM, all clones form bacterial
maps which are sensitive to bacteriocins when
grown at temperatures between 30°C and 34°C.

Above 34°C, bacterial mats no longer
form. This has also been observed in liquid LB
medium.

Sensitivity to bacteriocins is also
found for IPTG concentrations of only 0.15 mM
and at a temperature of 37°C. Therefore, there
exists a level of expression for genes iut A and
fep A, in the absence of an induction of vector
p GTI 001.

Morphological study.

The various clones undergo morphological
changes following overexpression of Iut A and
Pep A.

Bacteria, when observed with a phase
contrast optical microscope after induction at
42°C during 4 hours, show a notable elongation
(up to 10 times the average length of a normal
E. _Escherichia coli_). However, the more striking

characteristic is the presence of one to several
intracytoplasmic inclusions inside each bacterial
body.

Inclusions as observed with the optical
microscope may be found again when one observes
with an electronic microscope after negative
coloring of bacterial sections grown at -42°C
during 4 hours.

These inclusions are peripherical and
adjacent to the inner face of the cytoplasmic
membrane.

3.Immunological properties of proteins
Iut A and Pep A

Immunogenicity of Iut A and Pep A

Iut A proteins extracted from outer
membranes of strains E. coli 15822, 15893, 16003
and recombinant strain Escherichia coli CMR 603
GTI P-2 are isolated by preparative polyacrylamide
gels and injected to rabbits according to the
above described protocol.

Changes in the title of anti-Iut A
antibodies secreted by each rabbit are assessed with
the ELISA method, taking as an antigen a "granules"
(precipitated proIut A protein) solution extracted
from a culture of the strain E. coli CMR 603 GTI
P-2.

The positive reference serum used
is a rabbit anti-Iut A protein serum of E. coli
15I1-K12, supplied by B. OUDEGA.

The same protocol is followed for proteins
Pep A extracted from membranes of strains E. coli
15822 and E. coli RWB 18 GTI P11.

in all ... the rabbits react to the injection of ... Iut A and Pep A by producing a high titer of an... s specifically directed against these pro...

- Antig... roperties of proteins Iut A and Pep A

The spec...ty of the various obtained antibodies is stud... owards several outer membranes preparations (Stra:... .coli 15022, 15393, CMK 603 GTI P-2, CMK 6... B-5 and RWB 18 GTI P-12). This study is ... using the "Western blot" method.

The four ...i-Iut A sera ... which are prepared, ... well as the standard anti-Iut A colV-K30 ser... pecifically recognize a 76 kDa protein i... ll outermembrane preparations from strains expres... g the aerobactin system. Whenever the (wild ... recombinant, Iut A protein used for their ind... on, the antibodies of one serum specifically ... ognize the Iut A protein expressed by bird ... ox E. coli proteins and the two Iut A prot... synthetized by the recombinant strains E. coli CMK 03 GTI P-2 and CMK 603 GTI B-5.

The pre... sor of the Iut A protein, protein proIut A ... also specifically recognized by all anti-Iut A ...a. (Immunoblots made with purified "granules ... onerated by strains CMK 603 GTI P-2 and CMK 6 ...TI B-5).

Protein ...p 2 is not recognized by anti-Iut A sera an... conversely, anti-Pep A sera do not recognize ... A proteins.

Cloning of genes _Iut A_ and _Fep A_ in expression vector GTI 001 allows one to generate large amounts of proteins Iut A and Fep A as well as their respective precursors. Proteins Iut A or Fep A and their precursors which are synthetized following the induction of the transcription by the growth of bacteria at 42°C, rapidly accumulate in the form of large cytoplasmic inclusions (granules) which are visible with a phase contrast optical microscope. Observation with the electronic microscope of section of induced bacteria reveals that these granules are closely joined to the inner face of cytoplasmic membrane.

One must observe the importance of the expression level of Iut A and Fep A precursors (an average of 25% of total proteins under non-optimized conditions). Mature proteins make up as much as 35% of the protein content of the outer membrane. This percentage may be considered as the upper limit for the integration of this type of protein in the outer membrane. As a comparison, proteins Iut A and Fep A expressed by the wild strains Escherichia coli 15022 represent together 30% of the outer membrane proteins. It thus appears that the total expression of proteins Iut A and Fep A, and their precursors, by recombinant strains according to the invention is much higher than their natural expression.

The characterization of a sensitivity towards cloacine DF 13 in clones expressing the Iut A proteins, and of a sensitivity to colicine B in those which express protein Fep A shows that

the synthesis and integration of these proteins
in the outer membrane take place normally.

The identity of proteins obtained by
genetic recombination with wild proteins is also
demonstrated by the recognition of these proteins
by antibodies directed against natural Iut A and
Fep A proteins. One will note that these antibodies
also recognize, with the same specificity, precursors
proIut A and proFep A into intracytoplasmic inclusions.

Antibodies induced by proteins Iut
A and Fep A as obtained by genetic recombination
specifically recognize in the same way proteins
Iut A and Fep A as expressed by various strains
of pathogenic Escherichia coli.

Overexpression of receptors Iut A and
Fep A by cloning of their genes on an expression
vector, allows one to obtain in an iron-rich medium
external membrane proteins which are functionnally
and antigenically identical to proteins expressed
by pathogenic bacteria during their in vivo multiplication.

The synthesis of proteins Iut A and
Fep A through genetic recombination thus has
many advantages :

- it allows one to obtain proteins
Iut A and Fep A in very large amounts, while
freeing from regulation by iron,

- the synthetised proteins are functionnally
and antigenically identical to the proteins as
expressed by pathogenic bacteria in their in vivo
multiplication and they induce the production
of antibodies,

-when used as an active principle in
a vaccine, they induce the production of antibodies

preventing the specific recognition by membrane
proteins regulated by iron, of the siderophores,
thus stopping the supply of iron and blocking
their multiplication in a host ; thus they allow,
one to prepare very useful vaccines to prevent
or fight infections including septicemiae.

Vaccines may also be preparated simply
from inactivated recombinant clones or from membrane
fragments obtained by lysis of recombinant clones
followed by purification, according to usual methods
for the preparation of vaccines based on surface
or wall antigens.

IV. PREPARATION OF IROMPS BY GROWING
IN IRON-RESTRICTED MEDIUM.

1) Strain : E. Coli 078 reference 15022 :
origine : chicken

2) culture :

- Medium :

ST medium + succinate (Simon E.H. and
Tesmann (1963) Proc. Natl. Acad. Sci. USA 50,
526-532)

. addition of lactoferrin (250 ug/ml)
to obtain a medium with an iron deficiency,

. or addition of $FeCl_3$, $5H_2O$ (40 uM) :
for an iron-rich medium.

- Culture

- passing 3 times the strain in a
iron-rich medium, this being followed by an adaptating
stage in a deficient medium before final culture
in a deficient medium,

- simultaneously, one proceeds to grow
the same strain in an iron-rich medium,

- the cultures are made at 37°C during 24 heures.

3) Analysis:

At end of growth, in each medium, one proceeds to the following operations :

- harvesting by centrifugation,
- collecting the pellet in 0.2 M Tris HCl Ph 8, and ultrasonication,
- centrifugation (5000 g 30 minutes)
- recentrifugation of supernatant (100 000 g, 1 hour),
- taking up of the pellet in Tris HCl (50 mM, pH 8), $MgCl_2$ (10 mM), EDTA 1 mM, Triton 100 (2%) and agitating during 20 minutes at 37°C,
- centrifugating one hour at 100 000 g then reextracting the pellet,
- washing the pellet / in demineralised water,
- analysing the pellet in polyacrylamide gel (PAGE SDS) under denaturating conditions (mercaptoethanol, SDS).

4. Result :

- Membranes of bacteria grown with lactoferrin : presence of two proteins, in large amounts, having apparent molecular weights 80000 da (enterobactin receptor Fep A) and 76000 da (aerobactin receptor Iut A),
- membranes of bacteria grown in iron-rich medium : no 76000 and 80000 da band.

V - PREPARATION OF VACCINES

Preferably, the active principles according to the invention will be associated, in the vaccines, with a conventional antigenic preparation in known human or animal antibacterial vaccines.

and notably in the case when one uses purified proteins.

These vaccines may show the active inventive principles in usual liquid vehicles for parenteral administration. They may include conventional, for example oily adjuvants.

The legends of figures 1 and 2, hereabove referred to. follow :

For figure 1 :

Control of the expression of the Iut A protein and of its precursor on two CMK 603 clones containing GTI-Iut A constructs.

PAGE-SDS pattern of total proteins of the clones:
1. E. coli CMK 603 GTI-001
3. E. coli CMK 603 GTI-Iut A (strain 16003)
4. E. coli CMK 603 GTI-Iut A (strain 15972)
2. Molecular weight references.

For figure 2 :

Control of the expression of the Fep A protein and of its precursor.

PAGE-SDS pattern of total proteins of the clones.
1. E. coli CMK 603 GTI 001
2. E. coli CMK 603 GTI-Fep A.

TABLE I

PATHOGENIC STRAINS		SEROTYPE	ORIGINAL ANIMAL	REFERENCE
E. coli	15 022	O 78	chicken	souchothèque AX
"	15 393	O 56	calf	"
"	15 972	O 2	chicken	"
"	16 000	O138	calf	"
"	16 003	O138	calf	"
E. coli KM 576		(p ColV-K30)Man		B. OUDEGA

Host strains E. coli _ K12   genotype   origin or reference

. C 600	F⁻	thr, thi, leu, lac Y, fhu A, sup E	(MACRIS et al)
. HB 101	F⁻	hsd (r⁻s, m⁻s), recA, ara, pro, lacY, gal, rps, xyl, mtl, supE	MACRIS et al
. EWB 18	F⁻	thi, proC, leu, trp, entA, fepA	et al (14)
. CMK 603	F⁺	thr, thi, leu, supE, rec BC, fhu A, lac Y, r⁻s, m⁺	Institut Pasteur
. 15525	F⁻		Souchothèque Px

Bacteriocines-producing strains

E. coli   1300   Colicin B producing   R. PORTALIER

Enterobacter clocae DF 13 S458

                  Cloacin DF 13 producing

                          B. OUDEGA

TABLE II

Name	Size (bases pairs)	Characteristics	Reference or origin
pBR 322	4363	Amp^r Tet^r cloning vector	J. BOLIVAR
pAT 153	3600	Amp^r Tet^r cloning vector	A. TWIGG
pSB 118	2692	Amp^r derived from pUC18 cloning vector	P. STRAGIER I. Pasteur Paris
pGTI 001	5780	Tet^r expressing vector	P. BRUNEAU I. Merieux
pABN 1	18300	Amp^r vector p Plac + 16.3 kb Hind III fragment of ColV-K30 bearing operon aerobactin	GROSS et NEILANDS 1
pWS 101	15300	Amp^r vector pBr 322 + 11 kb Hind III fragment of E. coli chromosome bearing genes entD, fepA, fes et entF	LAIRD et YOUNG 1

TABLE III

Day 0	Day 14	Day 28	Day 35
intradermic injection	intramuscular injection	intramuscular injection	intramuscular injection
125ug	125ug	125ug	250ug
Blood sampling 5 ml		Blood sampling 10 ml	

Day 42	Day 49	Day 56
intramuscular injection	intramuscular injection	
250ug	250ug	
Blood sampling 50 ml		Blood sampling 50 ml

WILD PLASMID

↓

Digestion Bam HI

Electroelution

Digestion Bam HI

LIGATION

Bam HI

AMPᴿ

AMPᴿ
TETˢ

Bam hI

↓

Selection   with cloacine

↓

CLONES AMPᴿ TETˢ CLOˢ
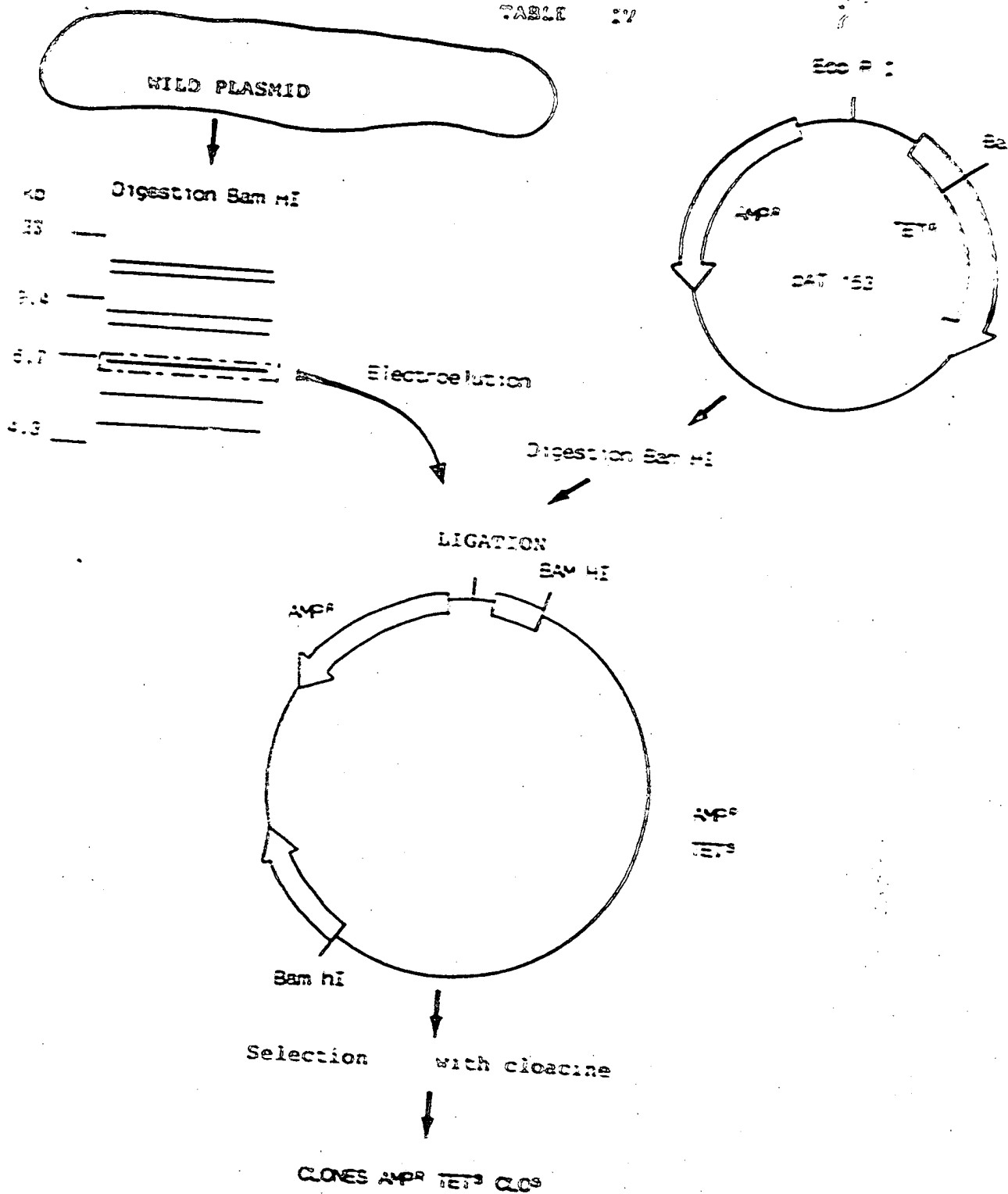
CLONING STRATEGY OF iut A GENES

TABLE V
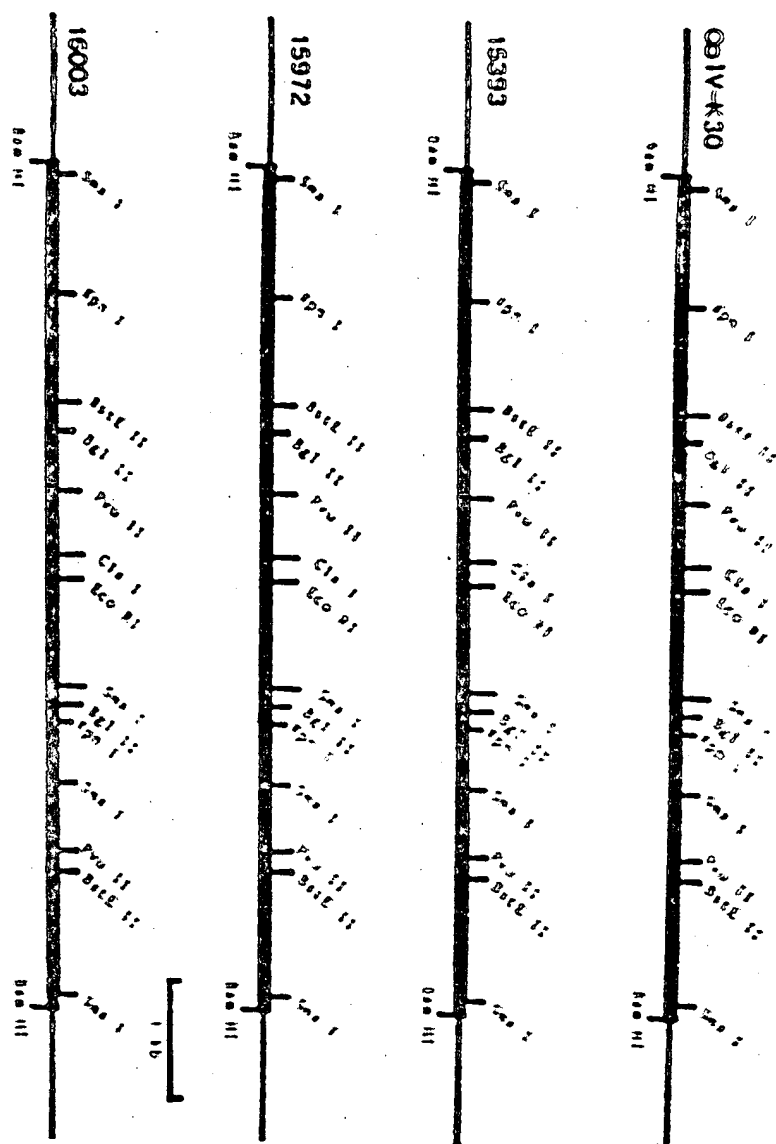
RESTRICTION MAP OF the HI -- Bam HI fragments bearing gene for a PROTEIN FROM STRAINS CoJV-K30, 15393, 15972 and 16003

Comparison between nucleotide sequences of BstE II – BstE II
(3.2 kpb) restriction fragments of *aerobactine operon*
of p CoIV-K30 et p 15972

ColV-K30
15972

SEQUENCING luc A

TABLE ... (continued)

TABLE VII (continued)

```
        AGATTACCGAACCCGAGCAC...  ...  ...
        CCAGATTA-TCGAACGCCAGCAGC...  ...
        ...CCGGTAAATATTATGGTCGCCCATT-...GTGTGGAGCGAACCT.CA
        ACCCGGTAAATATTATGGTCGCCC...T-TGTCGTGGACTCA...
        ...AACAAAGAGTGTGAACGT-ACTGACAGCAATTCAACGCTCAAGTT..
        ..ACAAAGAGTGTGAACGTCAGTGATGATCAATTTGAC.GTGAAACTTCA
        ..GCTGGTGGCGCTTACTGCAACAATTGCGTACCCAAACGGCGC.TT-
        .AGCTGGCTGGCGCTTACTGGCAACAATCGCGTACCCAAACGGCGCTTA
        A...TTGATAAGAGCGTGGTGGCGAATAAAGATCGACCATAGTGCGTGGA
        A...TTGATAAGAGCGTGGTGGCCAATAAAGATCGACGATTATCGTCGGA

CGAGAAA GCCGTATTACGGCGTGGAAGGTGCGGTGGACTACCTGATCCTGATACTGA
CGAT...CCCGTATTTACGGCGTGGAAGGTGCGGTGGACTACCTGATTCTGTACTCA
TTGA..TA.CGGATTCAAGTTCAACGTGCTGAAAATTGATTCAAAGTGAATGGTATCT
TTAT.  A.CCCATTCAAGTTCAACGTGCTGAAAATCGATTCAAAGTGAATGGTA.CTC
GCA  .TATCATGTGAAGACAGCAAGTTCATTAAAAGTCGACAGCTTA ATTGGTCTCT
GCAT -TATGATGTGAAGATAGCAAGCCATTAAAAGCCGACAGCTTATATTGGTCCCT
ACCC ..CCCTGGAGTTTGCGCGTGCAGAGTATCA.TTCCTTTGACGTGAGCTACCCCTA
ACCC .CCCTGGAGTTTGCGCGTGCAGAGTACCA.TTTTTTTAGCTTCTA  CA.CCCC
GGG.  .AAGGTCGATGGCTATACCACCGTGGATCTGTCCGCAGTTATCAGCTTCCCCT
GGG .AAGGTCGATGGCTATACCACCGTGGATCTGTCCGCATTATCAGCTT..CCT

GGGT ACTCAGCTTCAGCATTGAAAACCTTCGACTCGACTACACCACTGTTCGGG
GGG  ACTCAGCTTCAGCATTGAAAACCTTTGACTCGACTACACCACTGTTCGGG
GCA  TTGACCACTGTACTACAGCCCGGGTTATGGCCCAGCGTCACTGACGACTACAA
GCAT TTGACCACTGTACTACAGCCCGGGTTATGGCCCAGCGTCACTGACGACTACAA
AGT TTGGCC TACTTTGGTCGTGATCTACTGTGTGTGTGACCGGATCCTCCA
GT  TCCCAACTTTGGTCGAATACTGTGTGTGTGTGTGTCCGGATCCTCTA-
GAT AAGGTACGTCGATAATCAACATGGTCCCTCAAGTCAAGTTGATATCATATAATAT
GAT AAGGTACGTCGATAATCAACATGGTCCCTCATAGTCAAGTTGATATCATATAATA
A.  TTAATTTGTAGTTAGGGATGATAGACTTTATTGACTACAGATAATGTCGTGTT
A.  TTAATTTGTAGTTAGGGATGATAGACTTTATTGACTACAGATAATGTCGTGTT
```

TABLE III (continued)

BEGINNING 131

Matches = 3207   Mismatches = 10   Unmatched = -
Length = 3223   Matches/length = 99.5 percent

TABLE VIII

```
                '11
ColV-K30       GTG ATC TTC GGT GCG
'5393          ——————————————————
'5972          ————————C—————————
'6003          ——————————————————


                '148
ColV-K30       GAC GCA     TTC TGG CGG CTC AGT CGA TTA CGA TAC TTC ...
'5393          ——————C ATT CCT GGC GGC TCA GTC GAT TAC GAC AAC TTC ...
'5972          ——————C ATT CCT GGC GGC TCA GTC GAT TAC GAC AAC TTC ...
'6003          ——————C ATT CCT GGC GGC TCA GTC GAT TAC GAC AAC TTC ...


                '814
ColV-K30       ACC GAA CCG CAG CAG
'5393          ————————GC—————————
'5972          ————————GC—————————
'6003          ————————GC—————————


                2421
ColV-K30       GAA AAC CTC TTC GAC
'5393          ————————————T——————
'5972          ————————————T——————
'6003          ———————————————————


                2525
ColV-K30       GGC CCA CCT TTG GTC TGA
               Gly Pro Pro Leu Val END
'5393          GGC CGA ACC —— CGT CTG AAC TAC TCT GTG CTG TTC TGA
'5972          ——————GA ACC —— CGT CTG AAC TAC TCT GTG CTG TTC TGA
'6003          ——————GA ACC —— CGT CTG AAC TAC TCT GTG CTG TTC TGA
               Gly Arg Thr Phe Gly Leu Asn Tyr Ser Val Leu Phe END
```

Differences between the sequence of the three clones iut A and the sequence of gene iut A DE p ColV-K30

TABLE IX

Eco RI

CI857

5401
5125   Bam HI
Acc I

Eco RI

"TET"

PRм   OR3
           OR2
           OR1

P R

Shine and
Delgarno
de Cro

Bam HI

ATGGATCC

"Pr"

Bam HI 899

Xhol

ATG de Cro

"Ter"

p GTI 001
5780 pb

lac i

"Tac"   ORI

"Ter"
Bam HI 2388
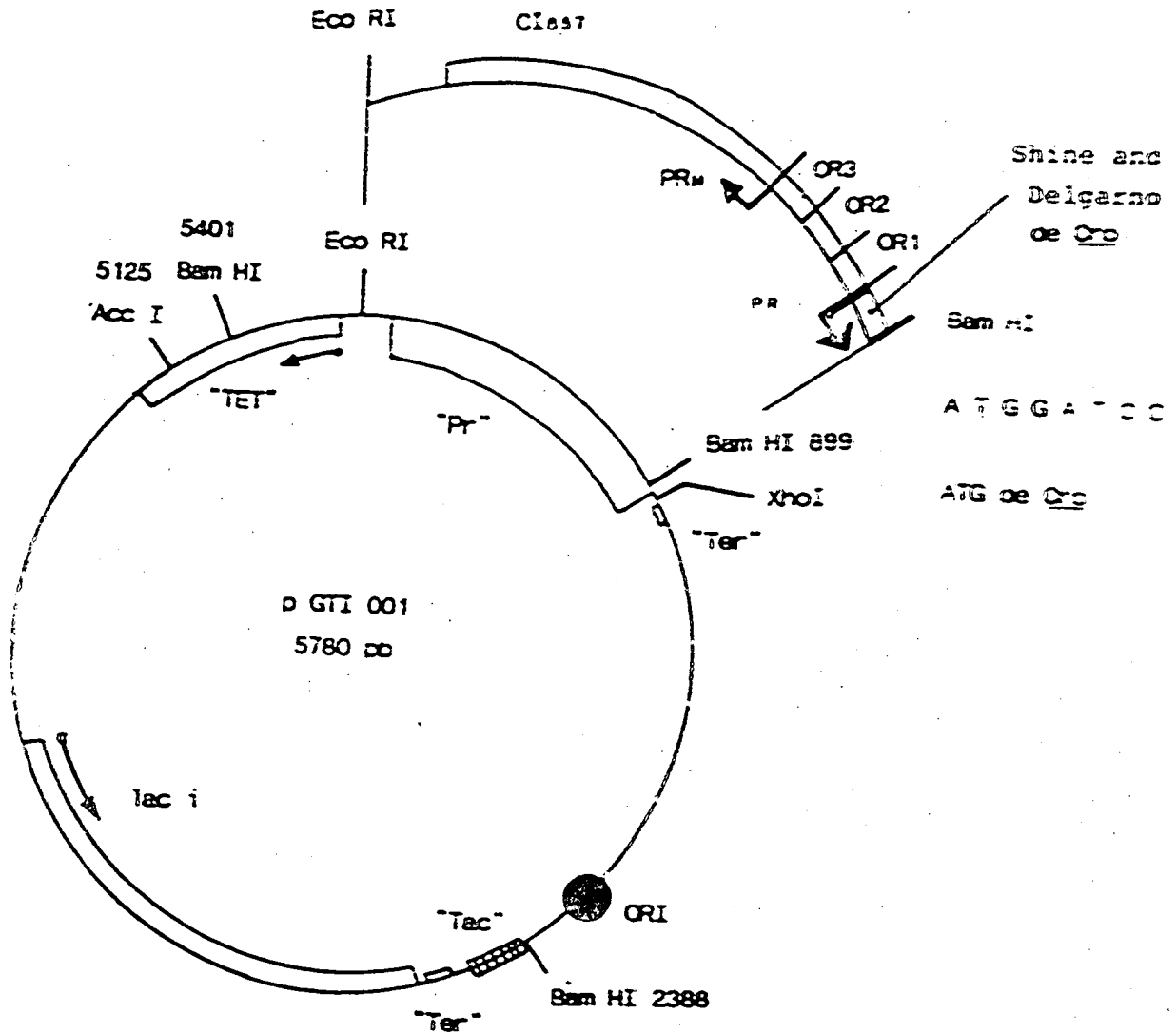
Creation of ATG

Digestion BamHi ... A T G G A T C C..
                   T A C C T A G G..

Digestion Mung Bean 5'... A T G 3'   >>>   5' A T G 3'
                   3'... T A C C T A G 5' >>>   3' T A C 5'

PHYSICAL AND GENETIC MAP of p GTI 001

TABLE X

ACC :

_____

ATG   ATG   ATA   AGC   AAA   AAG   TAT   ACG   CTT

MET   MET   ILE   SER   LYS   LYS   TYR   THR   LEU

1)   5' A T G A T A A G C A A A A A G T 3'

2)   3' T A C T A T T C G T T T T T C A T A 5'

5' A T G A T A A G C A A A A A G T 3'   oligo nucl
3' T A C T A T T C G T T T T T C A T A 5'

Start of gene _iut A_ and sequence of double strand
oligonucleotide used for the phasing of gene _iut A_ in p GTI 00

TABLE XI

Insertion strategy of
gene iut A in p GTI 001
1st part   DAT 153

Bam HI

Bst E II

10200 pb

Bam HI

Bst E II

vector DAT 153

Digestion Bst E II Removal band 3225 pb

Digestion Eco RV

KLENOW

Bam HI   Bam HI

ACC I

ACC I

8560 pb

LIGATION

(Eco RV)

Bam HI

Xhol

6900 pb

iut A

Eco R V

partial   digestion Bam HI

Mung Bean Nuclease

(Eco RV)

Digestion Eco RV

partial   digestion ACC I

ligation with olig. iut
double strand

Ligation by linker Xhol

(Eco RV)

Bam HI

Bam HI Eco RI (Bam HI) ATG

ACC I

ACC I

Pr

5

GTI P-2
8010 pb

iut A

Eco I

Digestion by Bam HI and Xhol and
removal   band 2780 pb

Xho I

Ligation with pGTI 001/partial
Bam HI/Xhol

Bam HI

TABLE XII

TABLE XIII

(Bam HI)

20

ACC I

830

Eco RI

Eco RI

379

Bam HI

276

ACC I

TET

Pr

lac A

380

p GTI P-2
p GTI B-5

7990 bp

Xho I

2737

1489

Bam HI

RESTRICTION MAP OF PLASMID S GTI P-2 and GTI B-5

TARGET DNA   Eco RI

Hind III

pBR 322   Bam HI

Sau I

Bam HI

Hind III   Seq A   Eco RI

p MS 101

15 500 bp

Sau I

Bam HI   Eco RI   Sau I

pBR 322

Bam HI

digested by

Digestion Bam HI

▼

LIGATION

▼

Bam HI

Sau I

PF-1

10600 bp

Eco RI

Bam HI   Sau I

Eco RI   Sau I
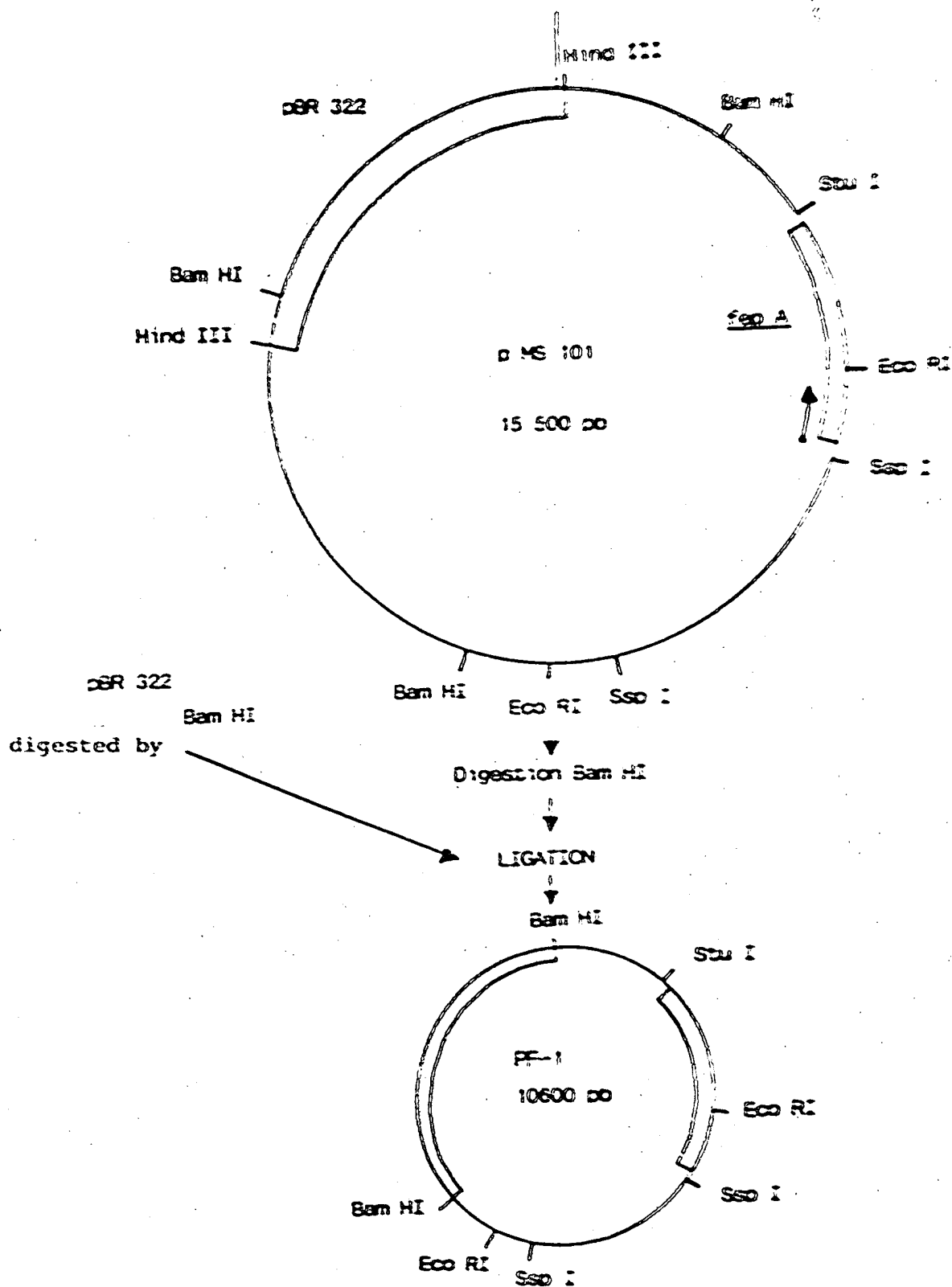
RESTRICTION MAP OF p MS 101 and CONSTRUCTION OF p F-1

TABLE XV

start of the sequence of gene _fep A_

```
                              ┌──┐┌──┐ ┌──┐┌──┐ ┌──┐
                              ┌─┐│  ││  │┌┐│  ││  │┌┐
                              │   ││  ││  ││   ││ ││  │││
5'   G G A A T A A A ACA A T G   A A C   A A G   A A G   A ~ ~ 3°

                                 MET   ASN   LYS   LYS   ILE
```
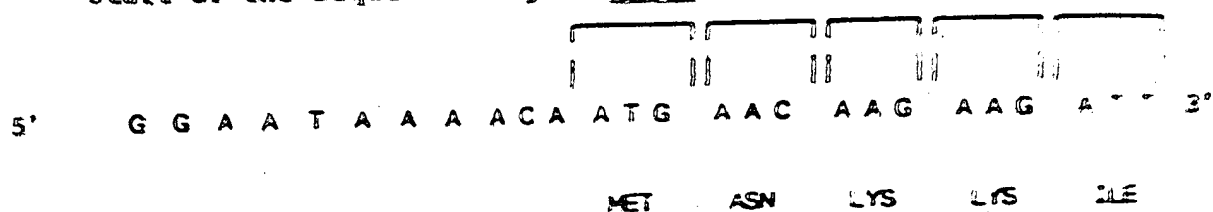
sequence to be obtained after mutagenesis :

```
5'    G G A A T A A A A C A G T T A A C A A G A A G A T T   3'

3'    C C T T A T T T T G T C A A T T G T T C T T C T A A   5'
                            │           │
                            └───────────┘

                         site Hpa I
```
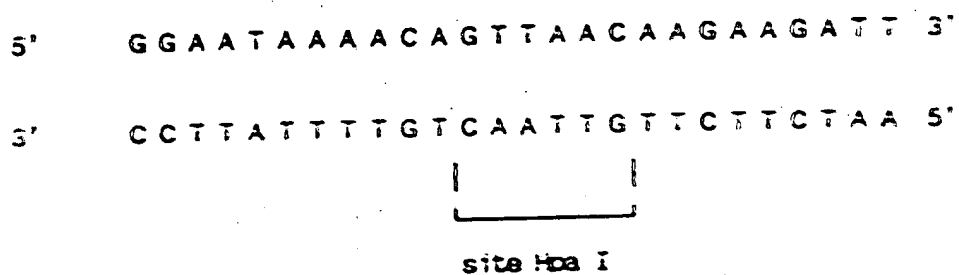
oligonucleotide (27 mer)
used

```
5'   C T T C T T G T T A A C T G T T T T A T T C C   3'
```

SEQUENCE OF SYNTHETIC OLIGONUCLEOTIDE USED FOR THE
MUTAGENESIS OP _fep A_

TABLE XVI
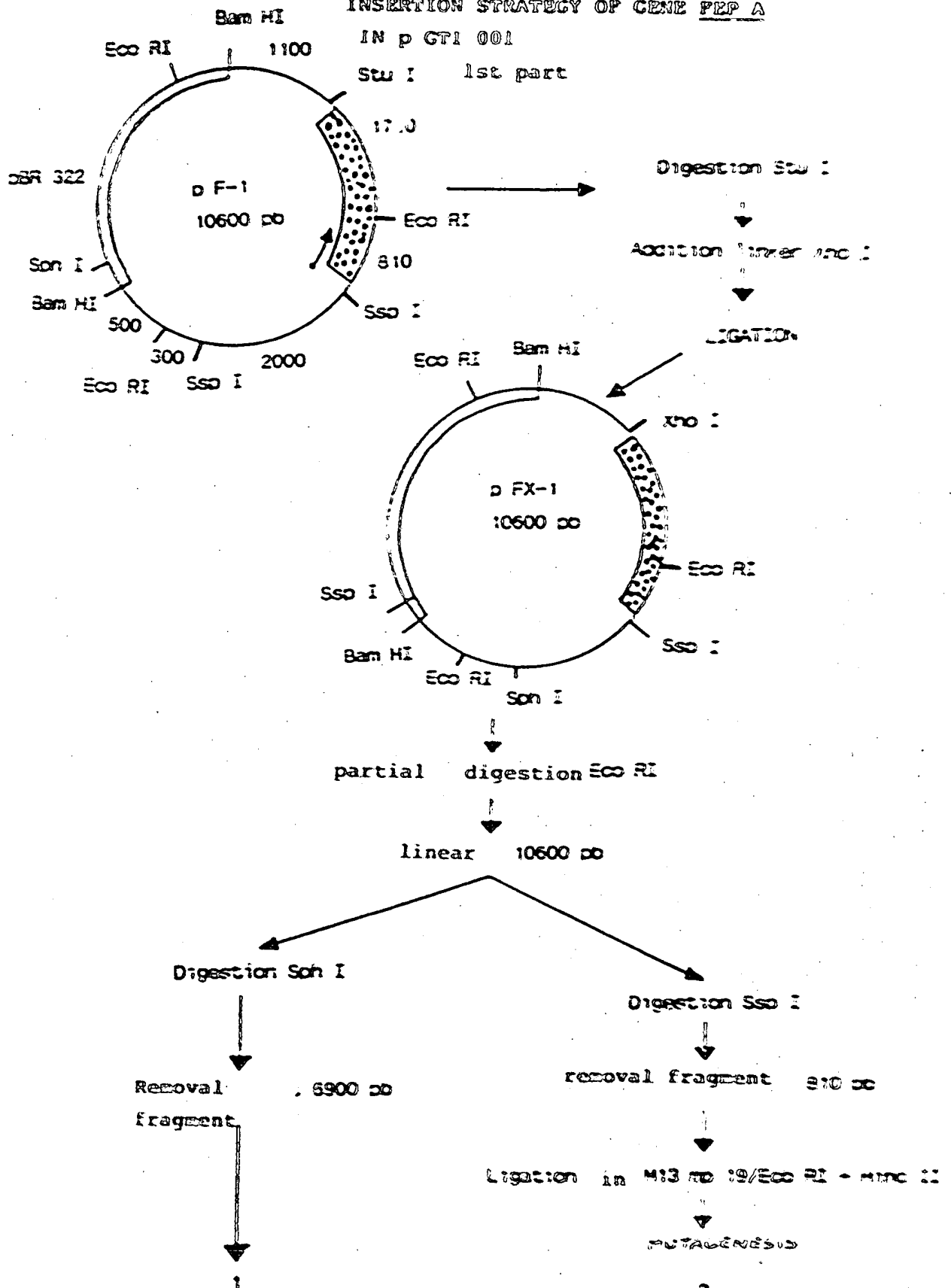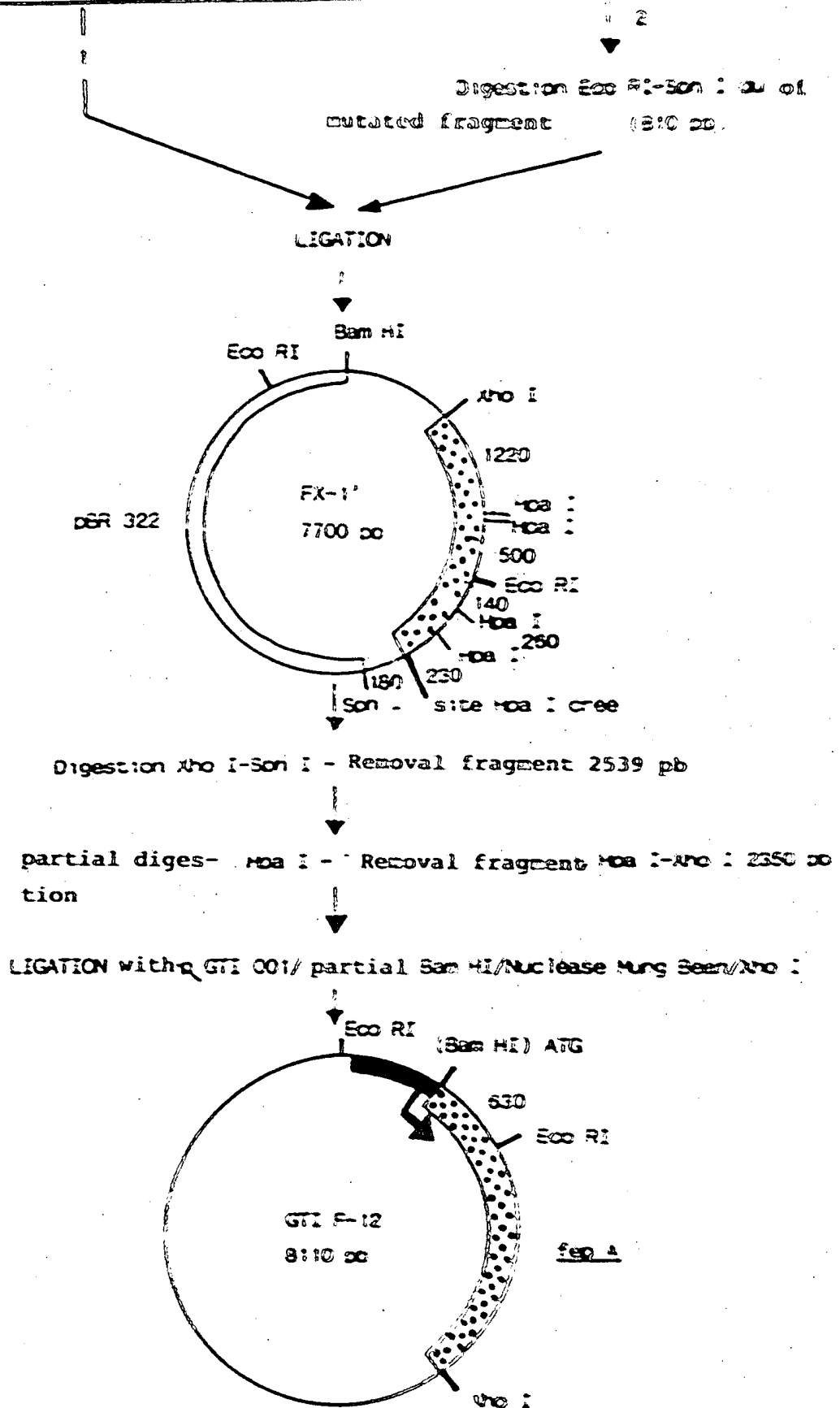### INSERTION STRATEGY OF GENE PEP A
IN p GTI 001

Stu I   1st part

Eco RI   Bam HI   1100

DBR 322   p F-1   10600 pb

Son I
Bam HI   500
300   2000
Eco RI   Sso I

Stu I   17.0
Eco RI
810
Sso I

Digestion Stu I

Addition linker Xno I

LIGATION

Eco RI   Bam HI

Sso I

Bam HI
Eco RI
Son I

p FX-1
10600 pb

Xno I

Eco RI

Sso I

partial   digestion Eco RI

linear   10600 pb

Digestion Son I

Removal fragment   6900 pb

Digestion Sso I

removal fragment   810 pb

Ligation   in M13 mp 19/Eco RI - Hinc II

MUTAGENESIS

1   2

TABLE XVII

Digestion Eco RI-Son I au of
mutated fragment (810 pb)

LIGATION

Eco RI — Bam HI

pBR 322

FX-1'
7700 pb

Xho I
1220
Hpa I
Hpa I
500
Eco RI
140
Hpa I
260
Hpa I
180 230
Son I - site Hpa I cree

Digestion Xho I-Son I - Removal fragment 2539 pb

partial diges-
tion
Hpa I - Removal fragment Hpa I-Xho I 2350 pb

LIGATION with GTI 001/ partial Bam HI/Nuclease Mung Bean/Xho I

Eco RI (Bam HI) ATG
630
Eco RI

GTI F-12
8110 pb

fep A

Xho I

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CLAIMS

1. A process for growing bacteria expressing outer membrane proteins regulated by iron, of which some are siderophores or transferrin receptors, which may be used in a vaccine preparation, characterized in that said bacteria are grown in a medium in which the iron content is reduced, with the help of a strong iron chelating protein, such as a lactoferrin, to a level allowing one to obtain an increased expression of said receptor proteins which is enough to induce, when said bacteria are used in a vaccine, the generation of antibodies preventing the specific recognition of siderophores by their receptors.

2. A process according to claim 1, characterized in that the bacteria belong to the group made up by enterobacteriae belonging to the families Escherichia coli, Klebsiella, Salmonella thyphimurium, Shigella.

3. A process according to claim 2, characterized in that the siderophores which are excreted by bacteria, are aerobactin and/or enterobactin siderophores.

4. A process according to claim 3, characterized in that the expressed proteins are Iut A and/or Fep A proteins.

5. An anti-septicemic bacteria vaccine characterized in that it includes, as an active principle, antigens comprising whole bacteria is obtained by culture in a medium wherein the iron content is reduced to a level allowing one to obtain an increased expression of outer membrane

proteins regulated by iron, and of which some
are siderophores or transferrin receptors, or
fragments of these bacteria, or transferrins
or siderophore receptors proteins, and notably
5   proteins Iut A and Pe A, extracted from these
bacteria.

6. A vaccine according to claim 5,
characterized in that said bacteria are bacteria
obtained by the process according to any of claims
10   1 - 4.

7. Bacteria expressing in increased
amounts at least one outer membrane protein, regulated
by iron and forming a transferrin or siderophore
receptor, characterized in that they include
15   recombinant expression vectors expressing said
protein(s).

8. Bacteria according to claim 7, characterized
in that the genes expressed in said vectors belong
to the expression system of the outer membrane
20   proteins regulated by iron inside the group
consisting of enterobacteria of the families E. coli,
Klebsiella, Salmonella thyphimurium, Shigella.

9. Bacteria according to claim 8,
characterized in that said proteins belong to
25   the systems of aerobactin and/or enterobactin
siderophores.

10. Bacteria according to claim 9,
characterized in that the synthetized proteins
are proteins Iut A and/or Pep A and/or their precursors.

30   11. Bacteria according to claim 10,
characterized in that protein Iut A is obtained
by a process wherein notably one isolates the
plasmid part of an E. coli strain or of any other
enterobacterium bearing the aerobactine operon,

one separates from the plasmid part a fragment
bearing gene <u>Iut A</u>, one ligates said fragment
with a cloning vector, one inserts the clones/gene <u>Iut A</u>
in an   expression vector, and one has protein Iut
A expressed by growing the clones.

12. Bacteria according to claim 11,
characterized in that the expression vector used
to express protein Iut A has its replication origin
located under the control of the tac promoter
and that the gene to express is under control
of the strong "Pr" promoter whose repressor is
thermosensitive.

13. Bacteria according to 10, characterized
in that protein Fep A is obtained by cloning gene
<u>fep A</u>   in the expression vector defined in claim
11 ou 12.

14. A process for synthesis of IROMPs
and notably proteins Iut A and Fep A or their
precursors proIut A and proFep A, characterized
in that clones as defined in claims 11-13 are
grown in an appropriate medium, first at a temperature
below 32°C then at 42°C to induce the expression
of genes <u>Iut A</u> and <u>fep A</u>.

15. Anti_septicemic bacterial vaccine,
characterized in that it contains as an active
principle IROMPs, and notably Iut A and/or Fep
A proteins and/or the precursors of these proteins
as extracted from the outer membrane or the cytoplasm
of recombinant bacteria, according to any of claims
7-13.

16. A vaccine according to claim 13,
characterized in that it contains said recombinant
bacteria or fragments of these bacteria.

1   2   3   4

94 kDa

76 kDa

67 kDa

43 kDa

30 kDa
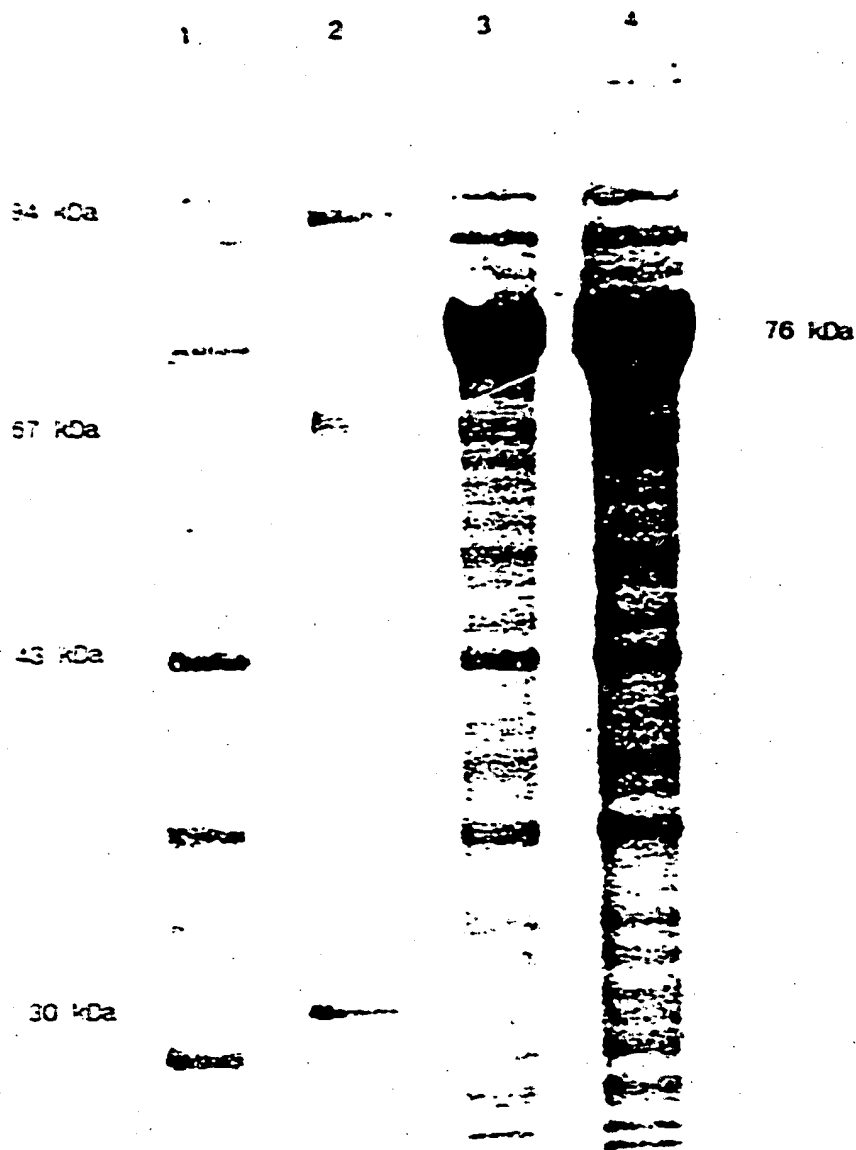
Control of the expression of protein Iut A and
its precursor in two CMK 603 clones with GTI-Iut
A constructions.

SDS-PAGE profiles of the total proteins in the
clones :

1. E. coli CMK 603 GTI-001

3. E. coli CMK 603 GTI-Iut A (strain 16603)

4. E. coli CMK 603 GTI-Iut A (strain 15972)

2. Molecular weight standards

Figure 47

Control of the expression of protein Fep A and
its precursor.
SDS-PAGE profiles of total proteins in the clones
1. *E. coli* CMR 603 GTI 001
2. *E. coli* CMR 603 GTI-Fep A